Proliferative activity, measured by MIB-1, and outcome in primary breast cancer following systemic therapy.

Pamela A Levack BMedBiol MBChB MRCP MRCGP

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Summary

There is increasing interest in the use of systemic therapy before surgery for large but operable breast cancers. It has the theoretical advantage of giving priority to the eradication of micrometastatic disease.

Studies of primary systemic therapy (PST) show that tumour regression rates are high and more conservative surgery is possible. The survival data look promising although the results of randomised trials are awaited. There is no uniform approach to the selection of PST. Combination chemotherapy is most frequently given and few researchers have used biological information from the tumour to help select therapy.

The Edinburgh study is an *in vivo* model of response to PST, examining tumour properties such as hormone receptor concentration and grade in relation to response and survival. Recent developments permit the use of monoclonal antibodies against proliferation associated antigens such as MIB-1 in archival material. This study relates MIB-1 to response to primary systemic hormone and chemotherapy and to survival.

Tissue was available for 65 patients treated between 1984 and 1988 and MIB-1 index was calculated in 61. Indices ranged from 0.01 to 0.89 and there was a significant ($p<0.002$) difference in mean MIB-1 between tumours classified as oestrogen receptor-rich ($\geq$20 fmol/mg) and oestrogen receptor-poor (<20 fmol/mg). MIB-1 expression correlated with increasing grade. There was a significant ($p<0.0004$) difference in MIB-1 between tumours which responded to primary hormone therapy and those which did not, and between those which achieved a complete pathological response following primary chemotherapy and those which did not (($p<0.01$). There was a significant ($p<0.005$) difference in survival between patients with high MIB-1 tumours treated with primary chemotherapy and those treated with second line chemotherapy after hormone failure.

Patients with ER-rich tumours which are highly proliferating may not respond to primary hormone therapy. A trial of hormone therapy may jeopardize subsequent response to chemotherapy and survival.

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Tables (continued)

Glossary

ABC system	Avidin-biotin-peroxidase complex
AMG	Aminoglutethimide
AJCC	American Joint Committee on Cancer
Complete pathological response	Absence of tumour on histological examination of the breast following PST
CHOP	cyclophosphamide 1gm/m^2, vincristine 1.4mg/m^2, adriamycin 50mg/m^2, prednisolone 40 mg daily for five days
DAB	Diaminobenzidine Identifies the site of an antibody by staining brown when incubated with peroxidase
DCC	Dextran coated charcoal assay to measure oestrogen receptor activity in fmol receptor sites/mg protein
Dose intensity	Amount of drug administered per unit time expressed as mg/m^2/week
Downstaging	The use of chemotherapy or hormone therapy to reduce primary tumour size or abolish oedema or erythema, altering tumour staging
Early breast cancer	Traditionally operable, T1/T2 with minimal node involvement Not a pure clinical or biologically sound term
EBCTCG	Early breast cancer trialist's collaborative group including representatives from 61 institutes
EORTC	European Organisation for Research and Treatment of Cancer
ER	Oestrogen receptor. Oestrogen binding protein MW 65,000, found in 50-60% of all breast cancers
FCS	Foetal calf serum
Growth fraction	Fraction of proliferating cells in a tumour
High risk operable disease	Stage I or II with heavy nodal involvement ± high grade
LABC	Locally advanced breast cancer. Equivalent to Stage 3

	(UICC AJCC 1983), which includes 3a (operable) T3N0 and Nl, 3b and T4 (inoperable). Includes inflammatory carcinoma - an aggressive subgroup of LABC with diffuse oedema and erythema frequently without a palpable mass
LHRH	Luteinising hormone releasing hormone. A decapeptide released from the hypothalamus which stimulates release of luteinising hormone from the pituitary
Mitotic figure index	Number of mitoses per 1000 tumour cells
NHL	Non Hodgkins lymphoma
NSABP	National Surgical Adjuvant Breast and Bowel Project
Proliferation associated antigens	Antigens which are detectable on growing or dividing cells ie in G_1, G_2, M and S phase
PST	Primary systemic (neoadjuvant) therapy
Recombinant	Different parts of genes combined together and re-introduced into the cell where it is reproduced along with the cells own genes
SPF S-phase fraction	Flow cytometric calculation of percentage of cells synthesising DNA
TBS	Tris buffered saline
UICC	Union Internationale Contra Cancer In 1985, together with AJCC agreed to a world wide TNM staging system

Proliferative activity, measured by MIB-1, and outcome in primary breast cancer following systemic therapy.

The concept of primary systemic therapy [PST] in breast cancer is not new and the ideas behind it have developed over the last century. Since Beatson[1] challenged the theory of parasitic infestation of the breast, hormone manipulation in advanced and metastatic breast cancer has played a major palliative role. In 1952 Schoenbach[2] demonstrated the value of chemotherapy in metastatic breast cancer using aminopterin, a folic acid antagonist with proven success in childhood leukaemia. In non-metastatic breast cancer, radical surgery often carried out as an emergency remained accepted surgical practice long after Haagenson[3] described the factors associated with inoperability. However the natural history of the disease suggests that in most patients surgical removal of the primary tumour is not curative.

The hypothesis that micrometastatic disease at presentation was responsible for treatment failure was confirmed experimentally by Schabel,[4] who showed in mice that chemotherapy given after excision of a C3H mammary tumour could prevent metastatic growth and be curative. Around the same time Fisher,[5] Bonadonna [6] and Nissen-Meyer,[7] pioneered the use of adjuvant chemotherapy. These trials, plus many more, have recently been subject to metanalysis [8] which has revealed an improved survival for all patient groups at ten years. It is now clear that the natural history of operable breast cancer is altered by early chemotherapy or hormone therapy.

Interest then focused on systemic therapy for local disease with a high tumour burden. Stage 3 (UICC) disease[9] is associated with a five year survival rate of 20%.[10-12] De Lena [13] was the first to question the mere supporting role of chemotherapy; he treated patients with inoperable Stage 3 disease with primary adriamycin and vincristine followed by radiotherapy. Tumour regression was achieved in 89%, dispelling anxiety about the chemosensitivity of large tumours with possible small growth fractions. Local control was ultimately accomplished with adjuvant radiotherapy in 97% of patients with non-inflammatory disease and a comparison with historical controls suggested improved survival at five years.

Numerous non-randomised studies of PST followed,[14-17] confirming high rates of tumour regression, often complete, achievable with combination chemotherapy. Downstaging rendered many "inoperable" tumours operable and allowed more conservative surgery. The effect of PST on survival is unknown, but five, ten and 15 year disease free survival figures of 53%,[18] 17%[19] and 28%[20] respectively, are encouraging and randomised prospective studies are in progress.

Studies of PST regularly included T3 N0 or N1 tumours, classified as Stage 3 because of their size, not their inoperability, and because primary tumour size is related to micrometastatic burden PST in high risk operable disease became a logical step.[21,22] The first UK study of PST for large operable breast cancers was in Edinburgh

from 1984 - 1990 [23] and the survival data are currently being analysed. In addition to the downstaging effect, the monitoring of primary tumour behaviour provided a unique in vivo model of response by which to examine tumour properties before and after therapy. Also, residual disease was surgically excised, permitting pre- and post-treatment properties to be related to immediate regression.

In the Edinburgh study, choice of PST was based on the patient's menopausal and tumour oestrogen receptor status. Other studies have concentrated on chemotherapy alone and the contribution of hormones has received little attention. Early results from the Edinburgh study were encouraging and a randomised trial of pre- versus post-operative chemotherapy or hormone therapy was started in 1990.[24] Several other trials of similar design are in progress [25,26] and the NSABP B-l 8 - "one of the most biologically compelling in the 30 year history of the NSABP".[27]

What systemic therapy should be selected? It is useful to examine the rationale for treatment choice both in metastatic disease and in adjuvant therapy. In metastatic disease, ER positivity of the primary tumour is a potent predictor of hormone induced response, associated with objective regression or useful palliation in 50% of patients.[28] In the adjuvant setting however, the picture is less clear. From the EBCTCG overview, all patients treated with tamoxifen showed an advantage in terms of risk of relapse or death, irrespective of receptor status, implying that ER negative micrometastatic disease may not be completely hormone refractory. The therapeutic

index may be improved by chemoendocrine therapy as results from the adjuvant NSABP B-16[29] and the EORTC study of LABC [30] suggest but these results conflict with theoretical arguments to support combined treatment.[31] The case for combined treatment is based on tumour heterogeneity and that against on the unfavourable effect of hormones on actively cycling cells. Accumulation of cells in non-proliferative phases of the cell cycle, as seen in vitro following LHRH agonists [32] and tamoxifen[33,34] and in vivo following tamoxifen,[35] may lessen the effect of chemotherapy.

Consequently it is not known whether ER positive tumours or a subset of these, would be better treated with chemotherapy initially or in combination with hormone therapy or whether some ER negative tumours should receive hormone therapy in addition to chemotherapy.

In selecting PST, it is important to decide which tumours should be treated and with what. Combinations of treatment and optimum dose should be considered. LABC is not uniformly fatal and up to 17% of patients have been recorded as surviving 20 years following surgery or radiotherapy.[12] These should be identified to prevent over-treatment. The value of a tumour model is that ineffective treatment may be identified quickly. In the Edinburgh [23] study a trial of hormone therapy was initiated but chemotherapy was substituted if the tumour did not regress.

Powles[36] found no overall difference in survival in a group of patients with metastatic disease treated with chemotherapy compared to untreated historical controls. More recently, correlations between dose intensity and response[37] rate of response and improved survival[38] and improved disease-free survival at two years following dose intensification - albeit when compared with historical controls[39] have been reported. This suggests that a subset of patients with metastatic disease may survive longer after high dose chemotherapy but care must be taken in extrapolating the results from metastatic to primary disease. The impact of high dose primary systemic chemotherapy is being examined in LABC with some promising early results.[40]

The choice of treatment for ER positive tumours is more difficult. The treatment of LABC in elderly patients with Tamoxifen is accepted practice. Regression is slow but it is unknown whether overall survival is any worse than if chemotherapy had been given. There is no clinical or pathological equivalent to ER for predicting chemotherapy response. Poorly differentiated and high grade tumours have been reported to show higher response rates to chemotherapy;[83] cellular proliferation, which has been valuable in improving treatment in experimental tumours, may have predictive value.

The Edinburgh large tumour study provides an opportunity to study the significance of cellular proliferation in vivo, in relation to both immediate response and to long term clinical behaviour. If tumour response is to have validity as an endpoint, it must be shown to correlate with overall survival. Although remission does not predict for

survival, data from PST studies suggests a correlation between rate of response and survival.[41,42]

Camplejohn[43] specifies criteria for a measure of proliferation in oncology. First it should have clinical value in different cancers. Second it should be technically simple to measure and neither too time consuming nor expensive.

The most widely used technique is S-phase fraction [SPF] using flow cytometric analysis which is strongly associated in most studies with clinical outcome of early breast cancer. High SPF generally[44-47] but not unanimously[48] correlates with poorer disease free and overall survival. It has been argued that SPF is a surrogate measure of grade but it is increasingly being reported as an independent prognostic factor.[49-50] It can be applied to fresh and paraffin- embedded tissue, and has been shown to identify patients retrospectively who benefited from combination chemotherapy as an adjuvant[51,52] and as treatment for metastatic disease.[53] Also in several small studies, regression following neo-adjuvant treatment correlates with SPF.[54-57]

Flow cytometry, however, is not technically simple. SPF cannot be calculated in up to 25% of samples[43,48] because of background debris or limitations in mathematical modelling in multiploid tumours. Its prognostic value depends on the simplistic division into "low" or "high".[58]

Proliferation-associated antigens can now be identified using monoclonal antibodies. Ki-67 antibody is widely used to recognise a non-histone nuclear protein which is expressed on all proliferating cells which are in the active parts of the cell cycle i.e. G_1, S, G_2 and mitosis but which is absent in G_0 cells.[59] Ki-67 status has been shown to correlate with grade in NHL[60] and breast cancer.[61-64] Associations between Ki-67 staining and recurrence,[65] survival,[66] response of metastases to chemotherapy[68] and response to endocrine therapy[67] have all been reported. Fresh tissue is required because the technique is unreliable using paraffin-embedded material.

Recently a method based on high temperature microwave heating of tissue sections has been described to retrieve antigens from formalin fixed paraffin-embedded tissues.[69] This unmasks the antigen, making it available for immunohistochemical staining and has increased the range of monoclonal antibodies which can be used to study archival material. Ki-S_1 is one such antibody which detects a proliferation antigen. Ki-S_1 status has been shown to have independent prognostic value[71,72] but the antibody is not commercially available. MIB-1 is a murine antibody recently raised against recombinant parts of the Ki-67 antigen[73] which can be tested on routinely processed paraffin embedded tissue, using the antigen retrieval microwave method. It shows an identical staining pattern to Ki-67 on fresh material and correlates well with Ki-67 in paraffin embedded sections of NHL[74] and breast.[75,76] MIB-1 also correlates with SPF [77] mitotic figure index [84] and inversely with ER.[68] The technique appears to be a highly

reproducible indicator of cell proliferation in breast cancer and provides an opportunity to study growth fractions by simple immunostaining. MIB-l has also been found to be an important predictor of survival,[78] and to correlate indirectly with chemotherapy induced response in high grade sarcomas.[79] In addition to these promising findings, it is relatively inexpensive.

The present study investidgates MIB- 1 staining in primary tumours from patients in a PST study which has the longest UK follow up data. MIB-1 values were ralated to patients survival and the relation between pre-treatrnent MIB-1 staining and tumour regression following hormone and chemotherapy was examined.

Patient population.

Patients and methods:

93 patients received either primary hormone therapy or chemotherapy within the Edinburgh large tumour study between July 1984 and August 1988.[23,24] A pre-treatment wedge biopsy or node biopsy was obtained for histological and biochemical examination at presentation. 66 of the original paraffin blocks were available from the archives of the Department of Pathology, University of Edinburgh, for MIB-1 immunohistochemical staining. Tumours had been originally fixed in buffered formaldehyde solution.

Systemic therapy was given for three months, after which a mastectomy or wide local excision was carried out. Pre-menopausal patients were treated by surgical oophorectomy or with LHRH analogue goserelin (Zoladex ICI 118630; 3.6mg subcutaneous depot preparation at 28 day intervals), and post-menopausal patients either with tamoxifen (20mg daily), aminoglutethimide (1000mg plus 40mg prednisolone daily) or 4 hydroxyandrostenedione (4-0HA Ciba Geigy CGP 32349; 250mg intramuscularly at 14 day intervals). Between 1984 - 1986 primary hormone therapy was prescribed irrespective of ER status but from 1987 it was reserved for patients with ER rich ($\geq$ 20 fmol/mg cytosol protein) tumours. If tumour progression occurred following hormone therapy, treatment was stopped and chemotherapy with CHOP started cyclophosphamide lgm/m^2, vincristine 1.4mg/m^2,

adriamycin 50mg/m^2 and prednisolone 40mg daily for five days) for four cycles at three weekly intervals. Patients with ER negative tumours or those with ER values< 20 fmol/mg were treated immediately with CHOP.

Tumour response was based on weekly tumour measurements by the author (PL) or her successor (Dr E Anderson). Three categories of response were defined. "Significant regression" and "significant progression" were defined as a 95% probability that the regression line of tumour volumes against time deviated from the horizontal. Response was classified as "no change" where the regression slope did not achieve this significance. The development of lymphoedema constituted progression.

Survival data was obtained by PL from patients' records and was available for all 66 patients.

Preparation of slides

New 4 μm sections from the original pretreatment paraffin blocks were cut unto poly-L-lysine slides. Tissue was processed for immunohistochemistry using the microwave antigen retrieval technique,[69] and immunostaining carried out according to the avidin - biotin (ABC) technique[70] with modifications by Cattoretti.[80]

Microwave processing.

Sections were de-waxed in xylene, rehydrated through a series of graded alcohols to 95% and incubated in 1% hydrogen peroxide for 30 minutes to block any endogenous breast tissue peroxidase. After washing, the slides were submerged in a solution of 0.01M Citric acid (pH 6.0) and microwaved for three periods of five minutes at maximum power in a 750 W microwave oven. Sections were washed in 0.05m Tris buffered saline (TBS) for five minutes and covered in 20% fetal calf serum (FCS) in TBS for ten minutes.

Immunohistochemistry.

Sections were then covered with MIB-l antibody (Immunotech., Marseille), diluted 1:50 in TBS/FCS for 30 minutes at room temperature and washed twice (x five min.) with TBS. Slides were covered with biotinylated rabbit anti-mouse antibody (1:100 dilution) for 20 minutes at room temperature, washed and covered with avidin biotin peroxidase complex (Dako Strept ABComplex, Denmark) for 20 minutes. Finally slides were soaked in a 1mg/ml solution of diaminobenzidine (DAB) containing 5% hydrogen peroxidase, washed and lightly counterstained with haematoxylin.

Quantitation of MIB-l staining

Representative fields from the most strongly stained areas of tumour were selected in collaboration with a Consultant Pathologist (Dr M. McIntyre, Western General

Hospital, Edinburgh). Using a 10 x 10 grid, at least 1000 tumour cells were counted at 400x magnification. MIB-1 antigen positive cells were identified by DAB brown staining, contrasting clearly with blue haematoxylin stained MIB-l negative tumour cells and an index was calculated.[81]

$$\textit{MIB-1 index} = \frac{\textit{number of MIB-1 positive cells}}{\textit{total number of tumour cells.}}$$

Statistical analysis.

Because grading is discontinuous, descriptive statistics are expressed as mode, median and range.

Survival was calculated using the method of Kaplan and Meier which allows for the use of censored data. Curves were drawn using an MS DOS computer programme "sureal" (Gregory personal communication).

Results.

Patient population.

The median patient age was 53 (range 38-69). Three were pathological grade 1, 15 grade 2, 33 grade 3 and ten were not graded. Clinical and pathological details are in Table 1. The distribution of ER status is in Fig 1 and was skewed with modal, median and mean values of 0, 36 and 83 fmol/mg respectively.

Immunohistochemical staining.

A MIB-1 index was calculated in 61 of 66 sections. In three cases, no tumour was identified in the block and in two there were insufficient tumour cells to calculate an index. Stain was present in both non-malignant and malignant cells. Non malignant proliferating cells found in the centre of germinal follicles showed strong nuclear staining (Fig 2a). MIB-1 staining of malignant cells demonstrated 2 patterns:

1. strong staining of nuclei, often in a particulate pattern as if identifying nucleoli.

2. at mitosis, chromosomal staining was very strong but diffuse cytoplasmic staining (Fig 2b) was also seen.

The percentage of cells which stained was very variable (1-89%). In some tumours less than 1% of malignant cells stained and these tumours often but not invariably were well differentiated and demonstrated gland formation (Fig 3b). In others most malignant cells were positive with a range of staining both in intensity and percentage of nucleus stained (Fig 3c). The distribution of MIB-1 (Fig 4) was approximately normal with mean and median values of 0.37 and 0.34 respectively. In order to examine the relation of MIB- 1 to other tumour properties, indices <0.4

were referred to as "low" and >0.4 as "high". There was no correlation between MIB-1 and ER (Fig 5) when both were assessed as continuous variables. However when ER values were divided into ≤20 or >20 fmol/mg (the criterion for clinical decision making) there was a significant difference ($p < 0.002$) in MIB-1 values between ER-rich and poor (Fig 5b). MIB-1 values >0.6 were only seen in ER-poor tumours, although approximately 25% of ER-rich tumours had MIB-1 values greater than average.

MIB-1 and grade.

59 of the 66 tumours had been graded previously by a pathologist (Dr Patterson, Department of Pathology, University of Edinburgh) as part of the large tumour study. MIB-1 expression was associated with increasing grade. The relation of grade(1-3) to MIB-1 was examined using the Mann-Whitney U test (Fig 6) and was significant between grade 1 and 2 ($p < 0.002$) and between grade 2 and 3 ($p < 0.05$).

Survival

There was no significant difference in survival between ER-poor (≤20 fmol/mg) and ER-rich (>20 fmol/mg) tumours (Fig 7) or low MIB-1 (<0.4) and high MIB-1 (≥0.4) tumours (Fig 8). Survival of patients treated with hormone therapy or chemotherapy alone was similar, the median survival not yet having been reached. The median survival of patients treated with chemotherapy after hormone failure was only 4.3 years.

Response to primary hormone therapy.

1. Primary tumour response.

The details of patients treated with hormone therapy are in Tables 2a and 2b. Those who responded to therapy are in Table 3 and those who progressed in Table 4. Mean MIB-1 values for responders and non responders are in Fig 9. A Mann-Whitney U test showed a significant ($P < 0.004$) difference in MIB-1 values between tumours which responded to treatment within 3 months and those which either failed to respond or progressed. 25/35 of ER-rich tumours had low MIB-1 values and 17 of these responded to primary hormone therapy (68%), and 10/34 ER-rich tumours had high MIB-1 values, of which 5/10 responded. No tumour with a MIB-1 value >0.5 responded to hormone therapy.

2. Survival.

As seen in Fig 10 the survival of patients treated with hormones alone was not significantly different from the survival of patients treated with primary chemotherapy. There was no difference in the survival of patients treated with hormones alone with respect to MIB-1 status (Fig 11). Nine of the 35 ER-rich patients had MIB-1 values >0.4. The behaviour of this group of ER-rich high proliferation tumours was compared to that of ER-rich low proliferation disease. The median survival of ER-rich high MIB-1 tumours was shorter than that of ER-rich low MIB-1 tumours (5 years v 9 years).

3. Relation of primary tumour response to survival.

Tumours which did not respond to primary hormone therapy and were treated with second line chemotherapy had a poorer prognosis. This difference was most evident for ER-rich tumours with high MIB-1 values. The median survival of this group of high proliferation rate tumours was only 3 years (Fig 12). Numbers are small and the difference is not significant but failure to respond to hormone therapy even when subsequent chemotherapy is given may be associated with a poor outcome.

Chemotherapy

1. Primary tumour response.

The details of patients treated with first line chemotherapy are in Table 5a. Five of these patients achieved a complete pathological response (Table 5b). A Mann-Whitney U test of the MIB-1 values of those achieving complete pathological response compared with those who did not was significant at a level of $p < 0.01$ (Fig 13).

The details of patients treated with chemotherapy after hormone failure are in Table 6 and the details of those who achieved regression and those who did not are shown in Tables 6a and 6b respectively. Only 1/17 patients achieved a complete pathological response. No change in tumour size was found in 6/17.

2. Survival.

Survival following chemotherapy (primary or secondary) was similar for high and low MIB-1 disease (Fig 14). Patients treated with primary rather than secondary chemotherapy appear to have an improved long term survival (Fig 15) but the difference is not statistically significant.

. When patients treated with primary chemotherapy only were examined those with the highest values had the longest survival but numbers were small and not statistically significance (Fig 16). However when patients with high MIB-1 levels were studied there was a significant difference in survival ($p < 0.005$) between patients treated with primary chemotherapy or with chemotherapy after hormone failure (Fig 17). Patients with high MIB-1 levels treated with primary chemotherapy had not yet reached their median survival and those who received hormone therapy first had a median survival of only 3 years.

Discussion

Study group.

The patients studied here were all treated within the first Edinburgh study of pre-operative systemic therapy for large operable tumours. They are therefore a selected group with considerable local disease and no clinically overt metastases, who are nonetheless likely to have micrometastatic disease. The ER distribution however was similar to that of an unselected group of patients presenting to the Edinburgh Breast Surgical Clinic:[82] the distribution was skew with a modal value of 0 fmol/mg, most of the very high (>100 fmol/mg) ER values occurred in post-menopausal patients and ER status increased with age.

The long term outcome of treatment was favourable - 50% of patients surviving ten years. This compares with a historical 10 year survival rate of less than 20% and rates of 31% and 17% in two of the most mature PST studies.[19,20] There was no difference in outcome between ER-poor and ER-rich tumours and 60% of patients with ER-poor disease were alive at 10 years. Neither was there any overall difference in outcome between patients treated with hormones alone and those treated with first line chemotherapy. As first line treatment with chemotherapy was based on ER negativity of the tumour, itself a poor prognostic factor in patients not receiving systemic therapy, it might be expected that this group would fare badly. In fact the median survival of ER-poor patients treated with primary chemotherapy and ER-rich patients treated successfully with hormone therapy has not yet been reached.

MIB-1

Tumours blocks were up to 11 years old and numerous sections had been taken previously, which may explain why no residual tumour was identified in 3 cases. It was relatively easy to distinguish positive brown DAB staining from blue haematoxylin staining although there was a range of staining intensity from weakly positive to strongly positive nucleii as has been previously noted.[78] Inflammatory infiltrates were not uncommon and co-operation with a pathologist was important to verify the denominator. The distribution of MIB-1 indices in the large tumours studied was approximately normal. The median (0.34) and mean (0.37) MIB-1 indices are higher than that those previously reported (0.25, 0.28) in a study which employed automated analysis to measure the percentage of total nuclear area staining.[78] The range of positivity (1-89%) is similar to that (0-80%) reported with the Ki-67 proliferation antibody.[65]

MIB-1 correlated with grade, a finding also previously reported[78] and one which is not unexpected as mitotic index is one component of grade. MIB-1 did not, as has been previously suggested,[68] correlate inversely with ER when both MIB-1 and ER were considered as continuous variables.This lack of correlation applied if the ER-poor and ER-rich tumours were considered separately. However the mean MIB-1 of ER-poor tumours was statistically significantly higher than that of ER-rich tumours. Increased MIB-1 expression, an indicator of tumour growth fraction, might be expected in ER-poor tumours but approximately 25% of ER-rich tumours also expressed greater than average MIB-1 levels.

MIB-1 alone was not predictive of long term survival, an association which has been previously demonstrated in patients with operable breast cancer who have received no systemic therapy.[78] Such associations have been regularly reported between survival and other measures of proliferation such as SPF and Ki-67. It is likely that any predictive value was negated by systemic therapy.

MIB-1 and response to hormones

No ER-poor tumour responded to hormone therapy and in the latter part of the study hormone therapy was only given as PST in patients with ER values >20 fmol/mg. ER values ≤ 20 fmol/mg therefore predicted for failure of primary tumour response. Values >20 fmol/mg did not guarantee response and although overall mean MIB-1 values were significantly higher in non responders than in responders (Fig 9) three tumours which progressed had higher than average ER and MIB-1 values (Table 7).

Patients with ER-rich high MIB-1 disease had a shorter survival. Four of the nine patients failed to respond to hormone therapy and all died within five years despite treatment with second line chemotherapy.

It is not possible to say whether the poorer outcome was due inherently to more aggressive disease or to the effects of treatment. These tumours may have been resistant to CHOP or the treatment sequence may have affected response. Tumours which failed to respond to one hormone therapy were treated with chemotherapy and alternative hormone therapy was not tried. It is unknown whether these tumours would fail to respond to all hormone therapies. There was (Fig 17) a statistically significant difference in survival between first and second line chemotherapy and this

was most evident in high MIB-1 disease (Fig 12). The chance of a mutation conferring drug resistance increases with each doubling therefore large tumours at presentation would be expected to contain such resistant clones already. In certain highly proliferating tumours any delay in effective treatment may jeopardize cell kill. Besides this time factor, hormone treatment may, in some rapidly proliferating tumours promote G_1 accumulation[32-35] and limit cell kill from subsequent cell cycle specific chemotherapeutic agents. It is unknown how long any such hormone induced effect may last.

MIB-1 and response to chemotherapy

Some large cancers, clinically localised to the breast, had high proliferation indices. Immediate treatment with combination chemotherapy, avoiding the delay caused by major surgery, resulted in a 50% ten year survival rate. Patients with the highest MIB-1 values of all fared the best although the numbers are as yet small. Patients who achieved a complete pathological response had significantly higher MIB-1 values than those who did not, and similar associations between response and pre-treatment proliferation have been reported for SPF[54-57] and Ki-67.[68] As seen above, patients with high MIB-1 values survived for a significantly shorter time if a trial of hormone therapy was given first.

In conclusion:

1. ER-poor disease predicted for failure of response to primary hormone therapy and patients unsuitable for primary hormone therapy could be selected on the basis of conventional tumour properties.

2. As in metastatic disease ER values ≥20 fmol/mg did not always predict for primary tumour response. Approximately 50% of ER-rich tumours progressed on therapy and some of these had ER values >200fmol/mg (Fig 18). Most ER-rich tumours had lower than average MIB-1 value. Approximately 25% in this study had above average values and tumours with values MIB-1 values >0.5 did not respond to hormone therapy.

3. MIB-1 index alone did not predict for hormone failure. However in a subset of patients with ER-rich tumours and high MIB-1 values unsuccessful hormone therapy predicted for a significantly poorer outcome. Long term survival was compromised by an ineffective course of hormone treatment in patients with MIB-1 values >0.4. A trial of first line chemotherapy may be more appropriate. It is possible that earlier detection of failure to respond may allow chemotherapy to be started sooner but at present there are no way of detecting either early response or failure.

4. MIB-1 as a measure of proliferation may satisfy several of Camplejohn's criteria [43] of usefulness for a immunohistochemical method. It works on conventionally fixed material. Although there is a graduation of staining intensity, positivity can be fairly easily identified. Furthermore it may have a useful role in identifying patients with ER-rich disease who should be considered for primary chemotherapy.

Tables

The tables 1-6 summarise the clinical and biochemical data of the 61 patients studied. The abbreviations used are shown here.

Dx Surgery: date of diagnostic surgery (wedge biopsy or node excision)

Rx 1: first treatment.

1 = tamoxifen
2 = Aminoglutethimide
3 = LHRH
4 = Oophorectomy
5 = 4 OHA
6 = CHOP

Resp 1: first response

1 = complete pathological response
2 = complete clinical response
3 = significant regression
4 = no change
5 = significant disease

health:

1 = alive and well
2 = alive with local recurrence
3 = alive with distant recurrence
4 = dead from breast cancer
5 = dead from treatment related effects
6 = unrelated death

ER: oestrogen receptor concentration (fmol/mg protein)

MIB1: MIB-1 index.

no	age	Dx surgery	Rx1	Resp 1	Rx2	Resp 2	last seen	health	ER	MIB-1	grade
1	61	90985	1	3			161292	5	62	0.48	3
2	44	291287	6	4			91294	1	6	0.8	3
3	47	30387	3	4	6	4	101094	3	30	0.02	1
4	59	180786	3	4			150990	4	73	0.4	ng
5	60	181286	3	5	6	3	111089	4	259	0.58	3
6	54	100387	5	5	6	4	230489	4	24	0.28	3
7	63	151087	5	3			130495	2	142	0.18	ng
9	63	240287	5	4			10691	4	225	0.07	2
10	57	40386	2	3			51293	4	381	0.18	3
11	61	140984	3	3			10595	3	158	0.16	2
13	53	120588	6	4			120894	1	17	0.41	2
14	48	250387	3	5	6	2	110789	4	0	0.71	3
15	61	250785	2	4			140994	1	167	0.16	3
16	53	190488	5	5	6	4	241094	3	149	0.3	2
17	66	300186	2	3			130694	1	147	0.29	3
18	49	40888	6	4			281194	1	8	0.52	3
19	45	30485	4	4			241094	1	93	0.17	3
20	65	250984	1	3			221193	4	221	0.25	3
21	52	10486	3	4	6	4	51188	4	2	0.2	3
22	43	10786	3	3			90790	4	70	0.33	ng
23	48	120886	3	3			51294	1	88	0.16	ng
24	41	130585	4	3			291189	4	50	0.4	2
25	60	40485	1	2			51286	4	45	0.04	ng
26	41	200887	6	2			230689	4	5	0.55	ng
28	50	11087	6	3			170894	4	15	0.01	2
29	59	270487	5	3			20994	1	344	0.36	3
30	54	90487	5	5	6	4	160489	4	266	0.47	2
31	46	150586	3	3			90990	4	36	0.23	2
32	51	190588	6	1			250794	1	8	0.82	3
33	52	80586	3	5	6	3	90589	4	320	0.51	3
34	42	230585	6	1			301194	1	0	0.67	2
35	65	81184	2	3			170394	1	148	0.41	ng
36	57	190985	2	5	6	1	170194	1	6	0.39	3
37	60	60685	1	5	6	3	10187	4	3	0.61	3
38	44	230186	1	5	6	2	140789	4	44	0.34	2
39	44	170387	3	3			51289	4	23	0.32	2
40	51	181087	6	3			140689	4	3	0.24	3
41	58	121285	6				11087	4	0	0.26	2
42	61	20485	6	3			190894	2	0	0.37	3

Table 1. Clinical and pathological details of patients in whom MIB-1 was measured.

no	age	Dx surgery	Rx1	resp1	Rx2	resp2	last seen	health	ER	mibl	grade
43	65	50785	1	5			70794	1	4	0.67	3
44	51	250735	2	5	6	3	300195	1	3	0.63	3
45	41	150588	6	3			280294	1	0	0.37	3
46	53	110785	2	3			201294	3	174	0.28	3
47	60	50686	3	5	6	3	51194	1	53	0.26	2
48	67	80288	5	3			241093	4	221	0.02	1
49	53	130287	6	2			270395	1	0	0.53	3
50	53	120588	6	3			100294	1	4	0.67	3
51	39	120588	3	3			40894	1	46	0.2	2
52	38	160485	4	5	6	4	10487	4	6	0.68	3
53	55	70285	2	5	6	3	221294	1	0	0.28	3
54	48	170985	6	1	0		120288	6	0	0.6	3
55	53	40987	6	1	0		140294	1	0	0.89	3
56	63	151087	6	1	0		240394	1	3	0.43	3
57	44	300686	3	3			71194	1	30	0.45	ng
58	45	210385	6	4	0		251285	4	0	0.09	3
59	64	111084	1	3			70395	1	380	0.42	ng
60	58	230188	6	3			90594	1	19	0.37	3
61	47	250486	3	5	6	3	180494	1	13	0.05	2
63	53		5	3			240593	1	51	0.11	1
64	62	160888	5	3			50994	1	286	0.10	3
65	69	240784	2	4			11194	1	55	0.31	3
66	60	260488	5	4	6	3	70593	4	81	0.55	ng

Table 1. Clinical and pathological details of patients in whom MIB-1 was measured.

no	age	Dx surgery	Rx1	Resp1	Rx2	Resp 2	last seen	health	ER	MIB-1	grade
1	61	90985	1	3			161292	5	62	0.48	3
20	65	250984	1	3			221193	4	221	0.25	3
25	60	40485	1	2			51286	4	45	0.04	ng
37	60	60685	1	5	6	3	10187	4	3	0.61	3
38	44	230186	1	5	6	2	140789	4	44	0.34	2
43	65	50785	1	5			70794	1	4	0.67	3
59	64	111084	1	3			70395	1	380	0.42	ng
patients treated with tamoxifen								median	**45**	**0.45**	
10	57	40386	2	3			51293	4	381	0.18	3
15	61	250785	2	4			140994	1	167	0.16	3
17	66	300186	2	3			130694	1	147	0.29	3
35	65	81184	2	3			170394	1	148	0.41	ng
36	57	190985	2	5	6	1	170194	1	6	0.39	3
44	51	250735	2	5	6	3	300195	1	3	0.63	3
46	53	110785	2	3			201294	3	174	0.28	3
53	55	70285	2	5	6	3	221294	1	0	0.28	3
65	69	240784	2	4			11194	1	55	0.31	3
patients treated with AMG								median	**147**	**0.25**	
3	47	30387	3	4	6	4	101094	3	30	0.02	1
4	59	180786	3	4			150990	4	73	0.4	ng
5	60	181286	3	5	6	3	111089	4	259	0.58	3
11	61	140984	3	3			10595	3	158	0.16	2
14	48	250387	3	5	6	2	110789	4	0	0.71	3
21	52	10486	3	4	6	4	51188	4	2	0.2	3
22	43	10786	3	3			90790	4	70	0.33	ng
23	48	120886	3	3			51294	1	88	0.16	ng
31	46	150586	3	3			90990	4	36	0.23	2
33	52	80586	3	5	6	3	90589	4	320	0.51	3
39	44	170387	3	3			51289	4	23	0.32	2
47	60	50686	3	5	6	3	51194	1	53	0.26	2
51	39	120588	3	3			40894	1	46	0.2	2
57	44	300686	3	3			71194	1	30	0.45	ng
61	47	250486	3	5	6	3	180494	1	13	0.05	2
Patients treated with LHRH								median	**43**	**0.29**	

Table 2a. MIB-1 and ER in patients treated with primary hormone therapy - Tamoxifen, AMG or LHRH analogue.

no	age	Dx surgery	Rx1	Resp1	Rx2	Resp 2	last seen	health	ER	MIB-1	grade
19	45	30485	4	4			241094	1	93	0.17	3
24	41	130585	4	3			291189	4	50	0.4	2
52	38	160485	4	5	6	4	10487	4	6	0.68	3

patients treated by oophorectomy

no	age	Dx surgery	Rx1	Resp1	Rx2	Resp 2	last seen	health	ER	MIB-1	grade
6	54	100387	5	5	6	4	230489	4	24	0.28	3
7	63	151087	5	3			130495	2	142	0.18	ng
9	63	240287	5	4			10691	4	225	0.07	2
16	53	190488	5	5	6	4	241094	3	149	0.3	2
29	59	270487	5	3			20994	1	344	0.36	3
30	54	90487	5	5	6	4	160489	4	266	0.47	2
48	67	80288	5	3			241093	4	221	0.02	1
63	53		5	3			240593	1	51	0.11	1
64	62	160888	5	3			50994	1	286	0.10	3
66	60	260488	5	4	6	3	70593	4	81	0.55	ng
patients treated with 4OHA								median	**148**	**0.24**	

Table 2b. MIB-1 and ER in patients treated with primary hormone therapy - oophorectomy or 4OHA.

no	age	Dx surgery	Rx1	resp1	last seen	health	ER	mibl	grade
1	61	90985	1	3	161292	5	62	0.48	3
20	65	250984	1	3	221193	4	221	0.25	3
25	60	40485	1	2	51286	4	45	0.04	ng
59	64	111084	1	3	70395	1	380	0.42	ng
10	57	40386	2	3	51293	4	381	0.18	3
17	66	300186	2	3	130694	1	147	0.29	3
35	65	81184	2	3	170394	1	148	0.41	ng
46	53	110785	2	3	201294	3	174	0.28	3
11	61	140984	3	3	10595	3	158	0.16	2
22	43	10786	3	3	90790	4	70	0.33	ng
23	48	120886	3	3	51294	1	88	0.16	ng
31	46	150586	3	3	90990	4	36	0.23	2
39	44	170387	3	3	51289	4	23	0.32	2
51	39	120588	3	3	40894	1	46	0.2	2
57	44	300686	3	3	71194	1	30	0.45	ng
24	41	130585	4	3	291189	4	50	0.4	2
7	63	151087	5	3	130495	2	142	0.18	ng
29	59	270487	5	3	20994	1	344	0.36	3
48	67	80288	5	3	241093	4	221	0.02	1
63	53		5	3	240593	1	51	0.11	1
64	62	160888	5	3	50994	1	286	0.10	3
						median	**142**	**0.26**	

Table 3. MIB-1 and ER in tumours which responded to primary hormone therapy.

no	age	diagnostic surgery	Rx1	resp 1	Rx2	resp 2	last seen	health	ER	MIB-1	grade
14	48	250387	3	5	6	2	110789	4	0	0.71	3
53	55	70285	2	5	6	3	221294	1	0	0.28	3
37	60	60685	1	5	6	3	10187	4	3	0.61	3
44	51	250735	2	5	6	3	300195	1	3	0.63	3
43	65	50785	1	5			70794	1	4	0.67	3
36	57	190985	2	5	6	1	170194	1	6	0.39	3
52	38	160485	4	5	6	4	10487	4	6	0.68	3
61	47	250486	3	5	6	3	180494	1	13	0.05	2
6	54	100387	5	5	6	4	230489	4	24	0.28	3
38	44	230186	1	5	6	2	140789	4	44	0.34	2
47	60	50686	3	5	6	3	51194	1	53	0.26	2
16	53	190488	5	5	6	4	241094	3	149	0.29	2
5	60	181286	3	5	6	3	111089	4	259	0.58	3
30	54	90487	5	5	6	4	160489	4	266	0.47	2
33	52	80586	3	5	6	3	90589	4	320	0.51	3

median ER = **13**
median MIB-1 = **0.45**

Table 4. MIB-1 and ER in tumours which progressed following hormone therapy.

no	age	Dx surgery	Rx1	resp1	last seen	health	ER	MIB-1	grade
2	44	291287	6	4	91294	1	6	0.8	3
13	53	120588	6	4	120894	1	17	0.41	2
18	49	40888	6	4	281194	1	8	0.52	3
26	41	200887	6	2	230689	4	5	0.55	ng
28	50	11087	6	3	170894	4	15	0.01	2
32	51	190588	6	1	250794	1	8	0.82	3
34	42	230585	6	1	301194	1	0	0.67	2
40	51	181087	6	3	140689	4	3	0.24	3
41	58	121285	6	4	11087	4	0	0.26	2
42	61	20485	6	3	190894	2	0	0.37	3
45	41	150588	6	3	280294	1	0	0.37	3
49	53	130287	6	2	270395	1	0	0.53	3
50	53	120588	6	3	100294	1	4	0.67	3
54	48	170985	6	1	120288	6	0	0.6	3
55	53	40987	6	1	140294	1	0	0.89	3
56	63	151087	6	1	240394	1	3	0.43	3
58	45	210385	6	4	251285	4	0	0.09	3
60	58	230188	6	3	90594	1	19	0.37	3
						median	**3**	**0.48**	

a. patients treated with first line chemotherapy

no	age	Dx surgery	Rx1	resp1	last seen	health	ER	MIB - 1	grade
32	51	190588	6	1	250794	1	8	0.82	3
34	42	230585	6	1	301194	1	0	0.67	2
54	48	170985	6	1	120288	6	0	0.6	3
55	53	40987	6	1	140294	1	0	0.89	3
56	63	151087	6	1	240394	1	3	0.43	3
						median		**0.68**	

b. Patients treated with first line chemotherapy who achieved a complete pathological response

Table 5. MIB-1 and ER in a. patients treated with first line chemotherapy
b. those patients in a. who achieved a complete pathological response.

no	age	Dx surgery	Rx1	Resp 1	Rx2	Resp 2	last seen	health	ER	MIB-1	grade
36	57	190985	2	5	6	1	170194	1	6	0.39	3
14	48	250387	3	5	6	2	110789	4	0	0.71	3
38	44	230186	1	5	6	2	140789	4	44	0.34	2
5	60	181286	3	5	6	3	111089	4	259	0.58	3
33	52	80586	3	5	6	3	90589	4	320	0.51	3
37	60	60685	1	5	6	3	10187	4	3	0.61	3
44	51	250735	2	5	6	3	300195	1	3	0.63	3
47	60	50686	3	5	6	3	51194	1	53	0.26	2
53	55	70285	2	5	6	3	221294	1	0	0.28	3
61	47	250486	3	5	6	3	180494	1	13	0.05	2
66	60	260488	5	4	6	3	70593	4	81	0.55	ng

a. tumour response following chemotherapy. median **81** **0.42**

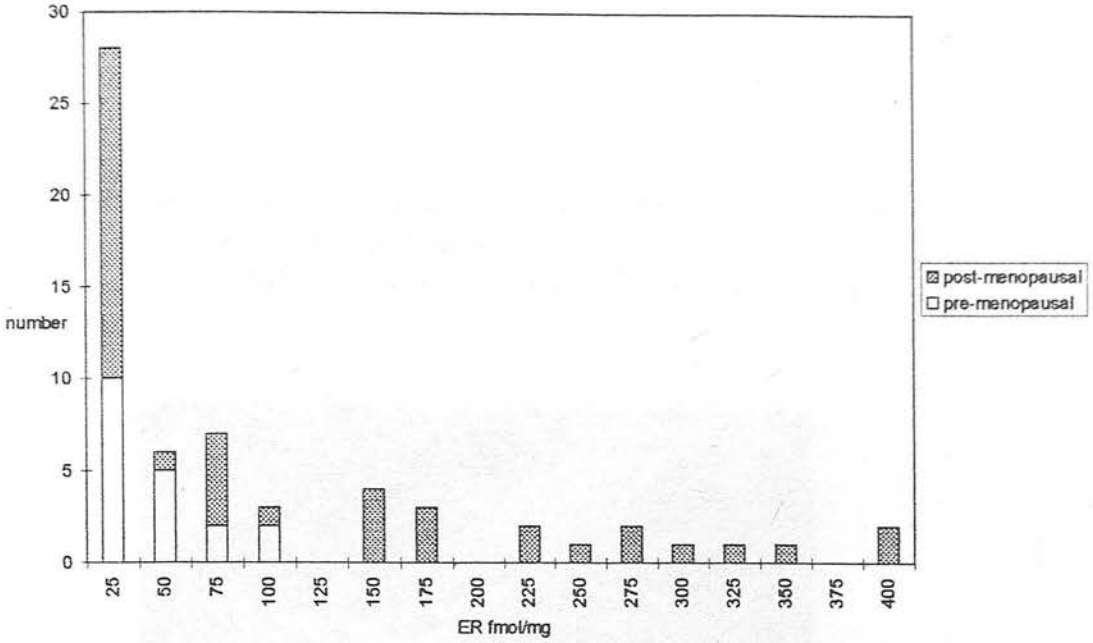
no	age	Dx surgery	Rx1	Resp 1	Rx2	Resp 2	last seen	health	ER	MIB-1	grade
3	47	30387	3	4	6	4	101094	3	30	0.02	1
6	54	100387	5	5	6	4	230489	4	24	0.28	3
16	53	190488	5	5	6	4	241094	3	149	0.3	2
21	52	10486	3	4	6	4	51188	4	2	0.2	3
30	54	90487	5	5	6	4	160489	4	266	0.47	2
52	38	160485	4	5	6	4	10487	4	6	0.68	3

b. no tumour response after chemotherapy median **27** **0.32**

Table 6. MIB-1 and ER in patients treated with 2nd line chemotherapy (i.e. after hormone failure).
a. tumour response after chemotherapy
b. no tumour response after chemotherapy.

	MIB-1 <0.4	MIB-1 >0.4	TOTAL
ER +	0.26,0.28,0.29, 0.34 **(4)**	0.47,0.51,0.58 **(3)**	7
ER -	0.05,0.28,0.39 **(3)**	0.61,0.63,0.67, 0.68, 0.71 **(5)**	**8**
			15

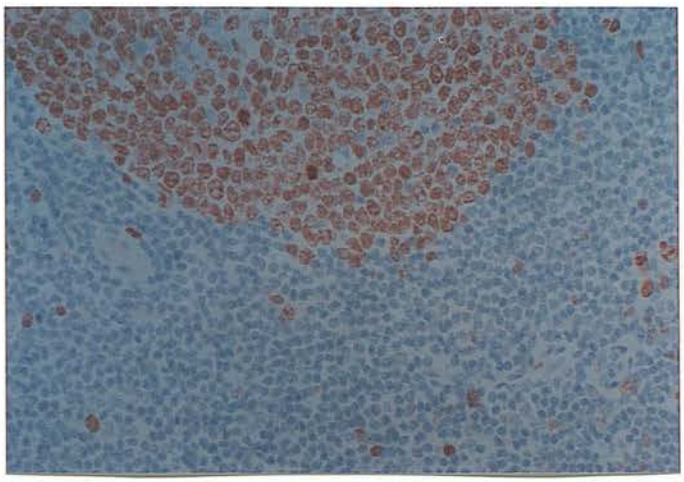
Table 7. MIB-1 values of patients treated with hormone therapy in whom the primary tumour progressed.



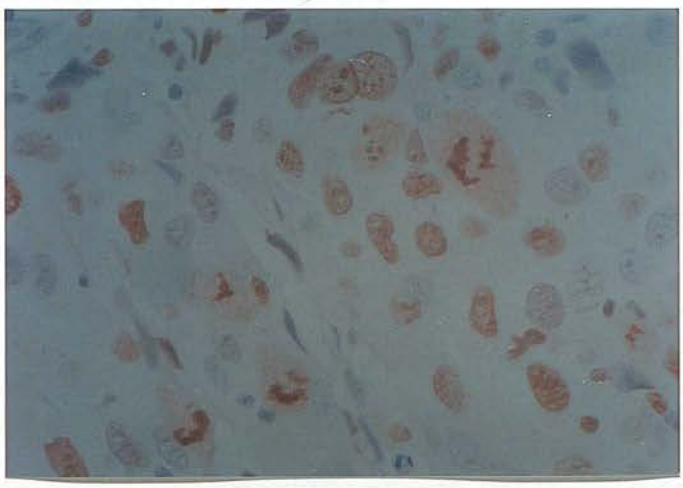
n = 61
median = 36
mean = 83

Fig 1. The distribution of ER in all patients studied.

Fig 2. Immunostaining of microwave processed paraffin sections of
a. normal lymph node follicle
b. mitotic figure showing nuclear and cytoplasmic staining



a. Normal proliferating germinal centre cells stain strongly.

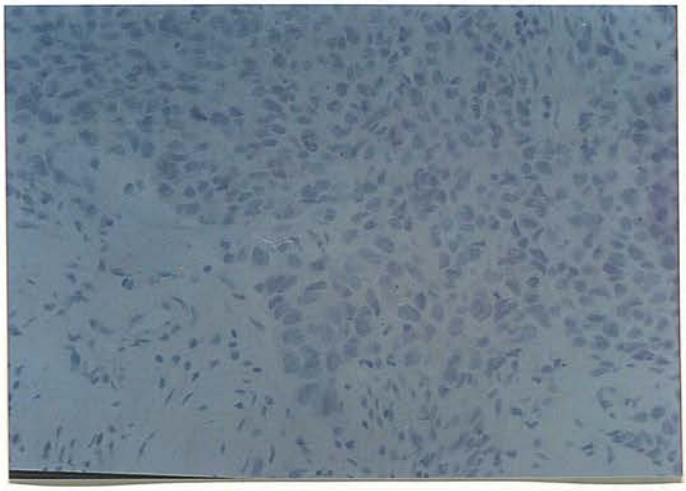


b. MIB-1 staining at mitosis

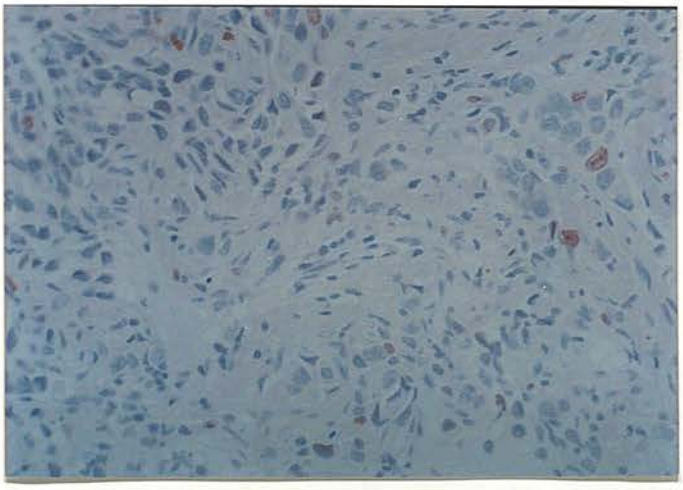
Fig 3 Immunostaining of microwave processed paraffin sections with MIB-1 staining .

a. invasive ductal carcinoma - control
b. invasive ductal carcinoma low MIB-1 staining
c. invasive ductal carcinoma high MIB-1 staining

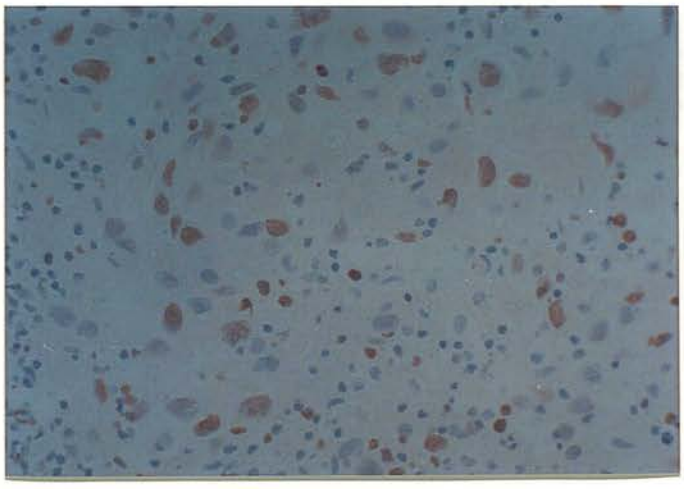
a.

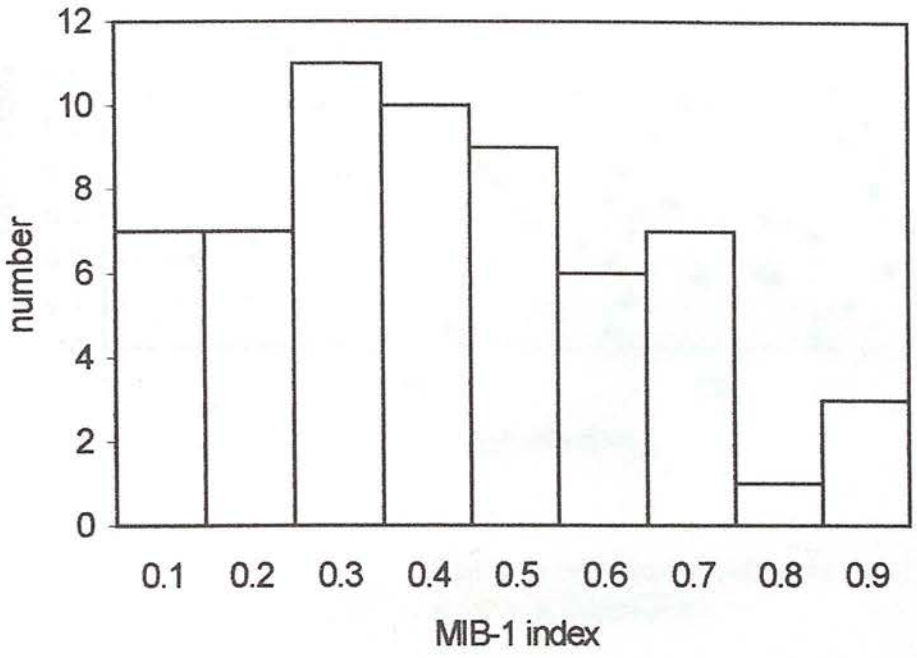


b.



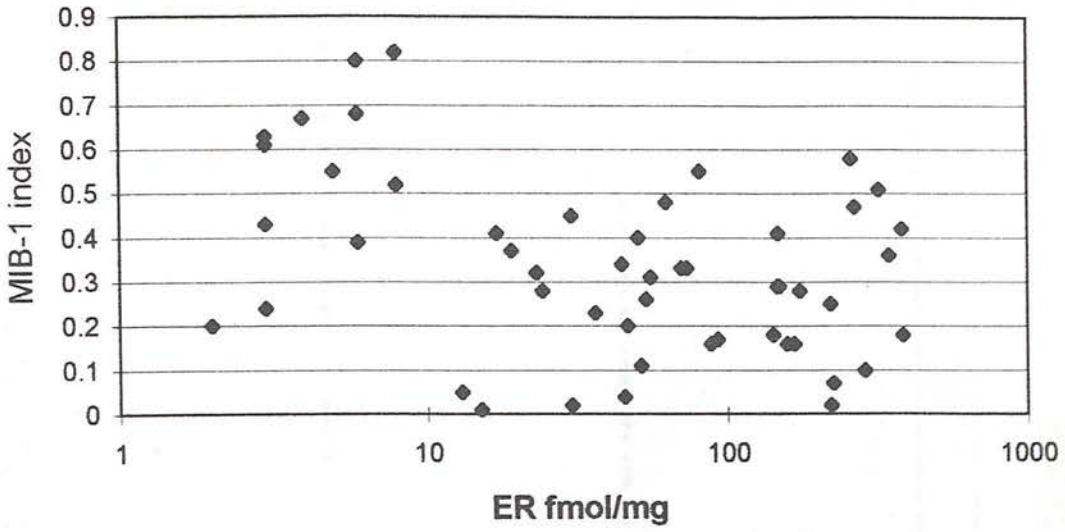
c.





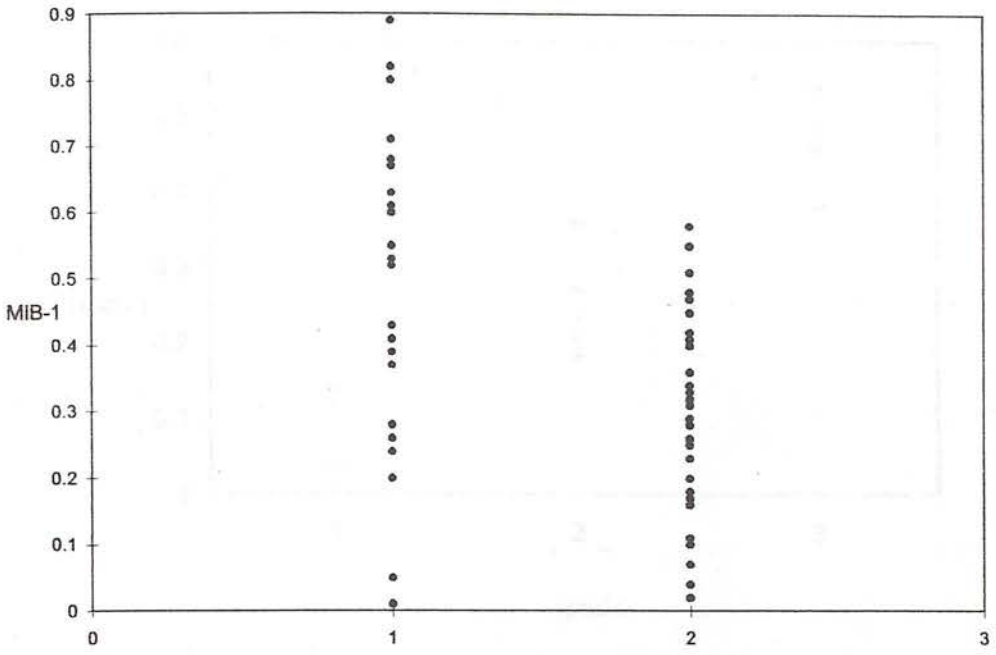
n = 61
median = 0.34
mean = 0.37

Fig 4. The distribution of MIB-1 in all patients.



Pearsons correlation coefficient = -0.27
p >0.2 not significant

Fig 5. The correlation between MIB-1 and ER in all patients studied



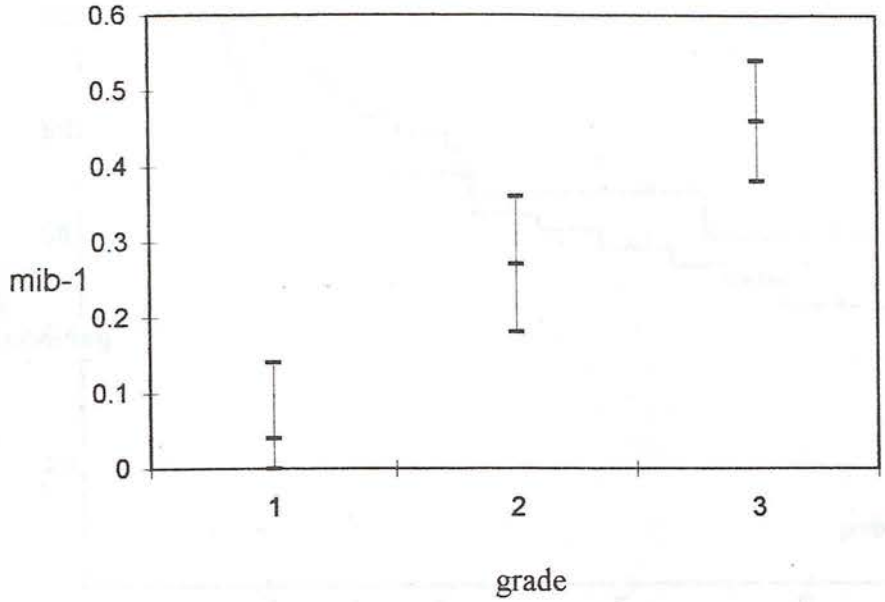
ER <20 fmol/mg ER ≥ 20fmol/mg

mean MIB-1 ± 95% confidence limits of mean

0.48 ± 0.08 0.25 ± 0.05

p = < 0.002 Mann-Whitney

Fig 5a. MIB-1 values in ER negative and ER positive tumours.



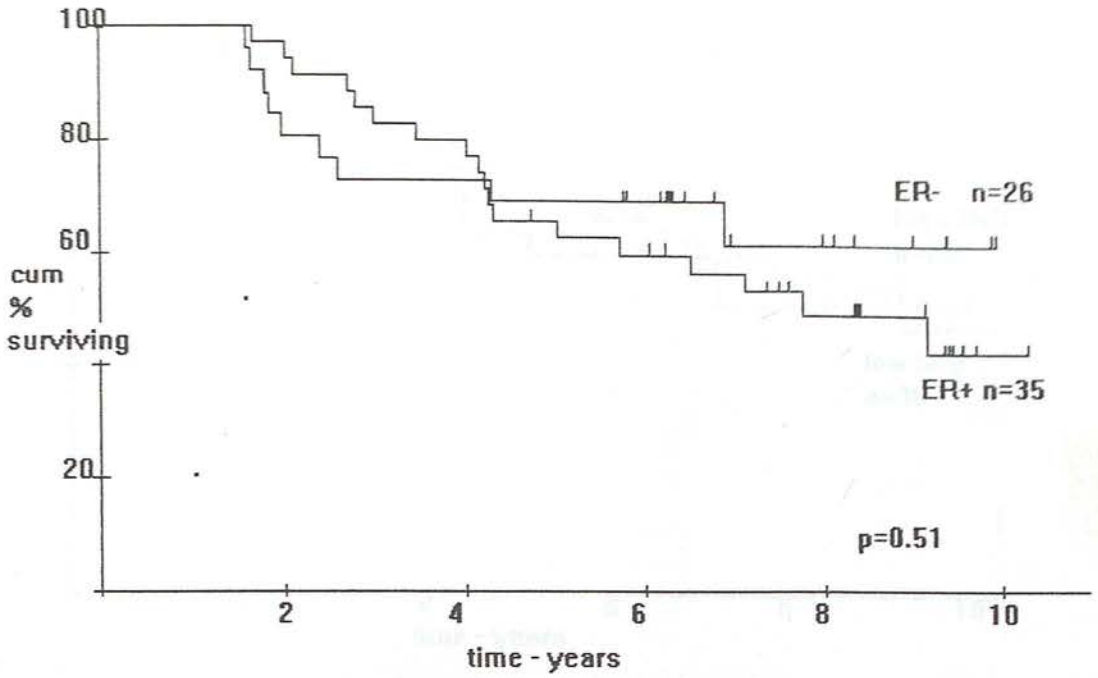
mean ± 95% confidence intervals of mean.

0.04 ± 0.1 0.27 ± 0.09 0.46 ± 0.08

$p < 0.002$ $p < 0.05$

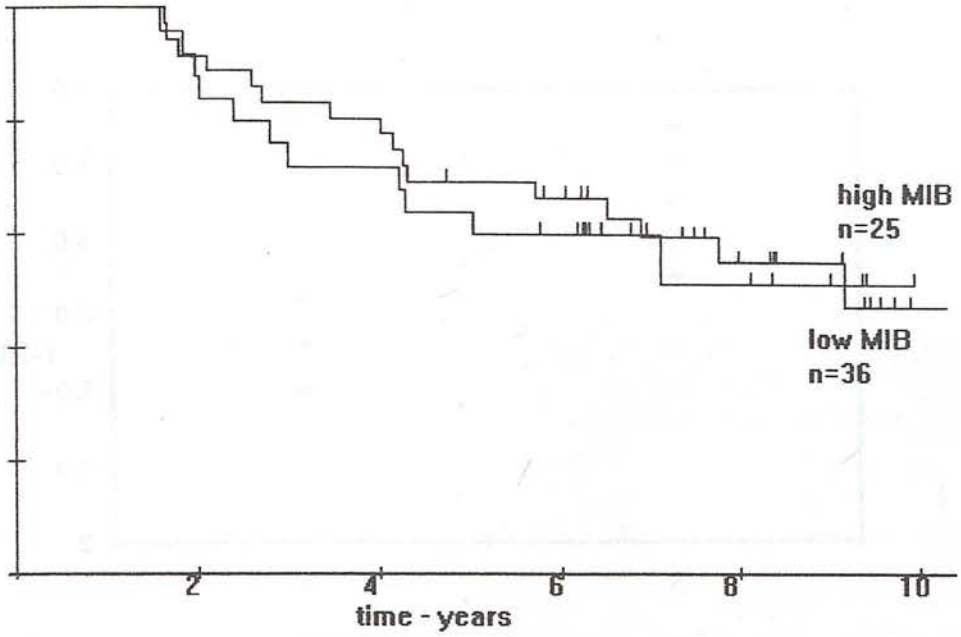
Mann-Whitney U test

Fig 6. The relation of MIB-1 to grade.



median survival ER-	not yet reached
median survival ER+	7.7 years

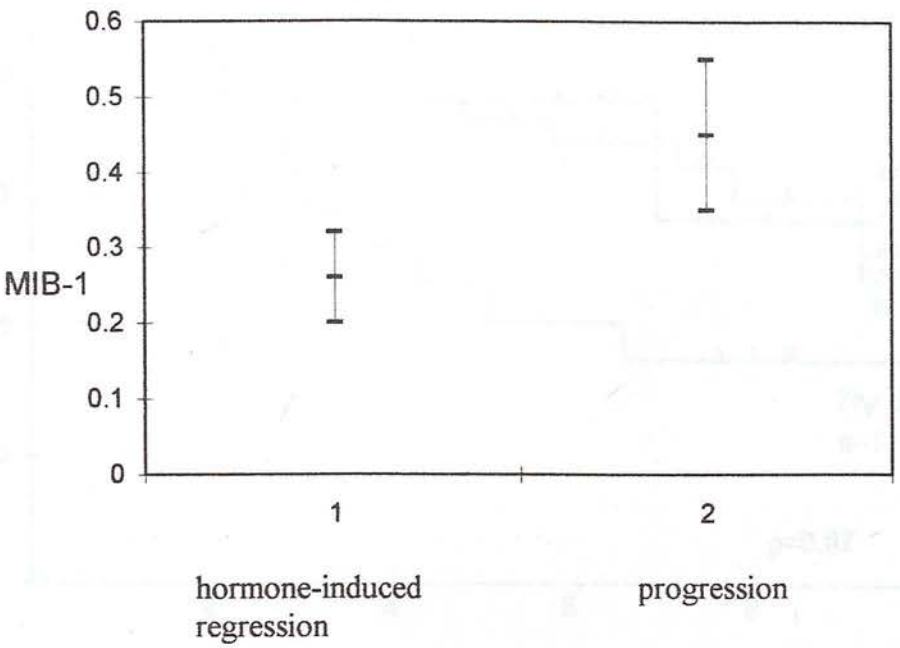
Fig 7. Survival in ER - (≤20 fmol/mg) and ER+ (>20 fmol/mg) disease.



y = cumulative % surviving.

median survival low MIB-1	9.6 years
median survival high MIB-1	not yet reached

Fig 8. Survival in low (<0.4) and high (≥0.4) MIB-1 disease.

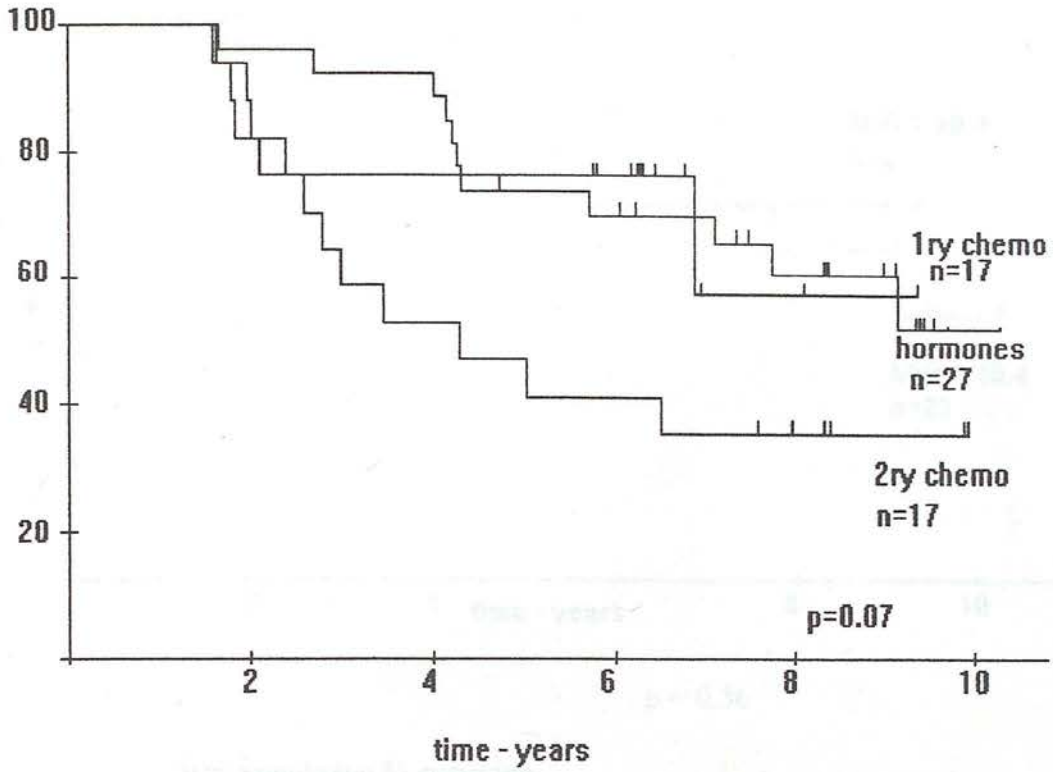


mean MIB-1 ± 95% confidence limits of mean

0.26 ± 0.06 0.45 ± 0.13

$p < 0.004$ (Mann-Whitney U test).

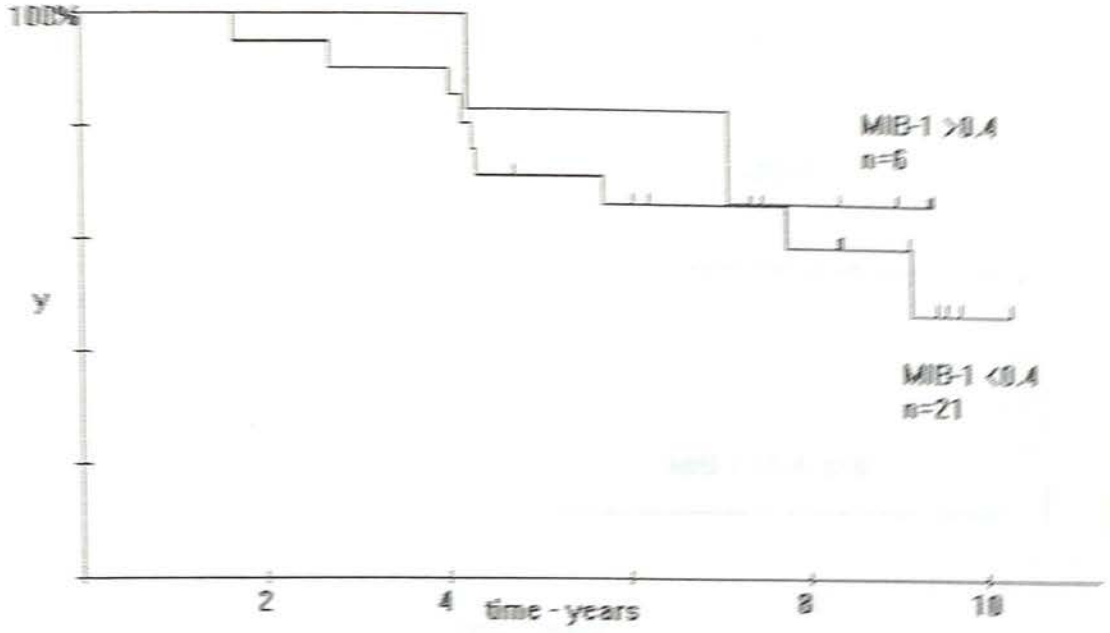
Fig 9. MIB-1 values in relation to primary tumour response following hormone therapy.



y = cumulative % surviving

median survival following:	hormone therapy	not yet reached
	1st line chemotherapy	not yet reached
	2nd line chemotherapy	4.3 years

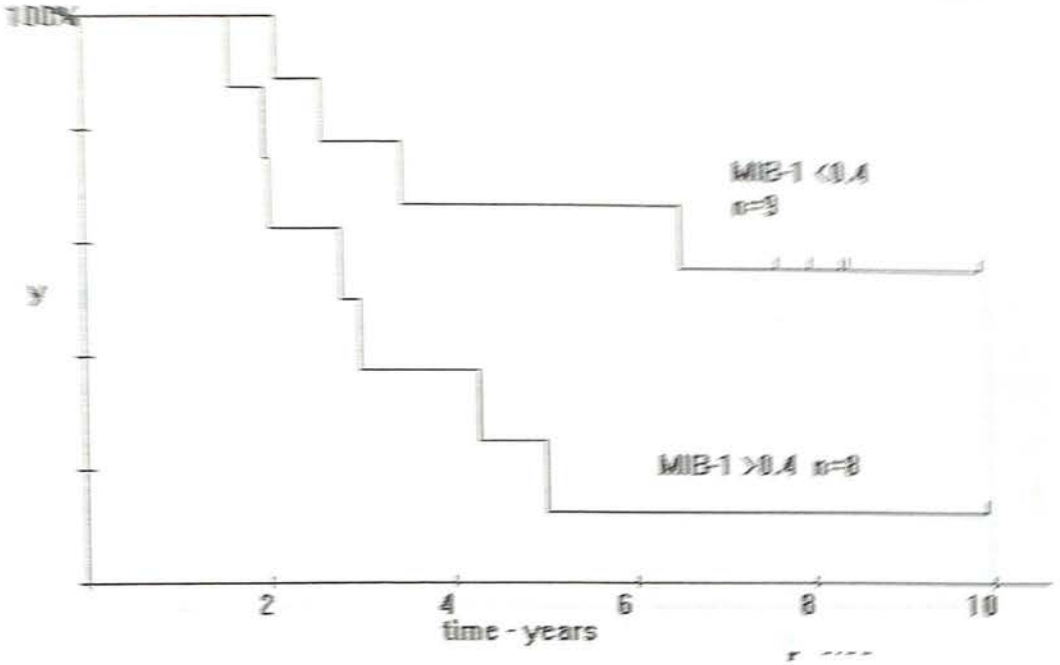
Fig 10. Survival of patients treated with hormone therapy alone, primary chemotherapy or chemotherapy after hormone failure.



p = 0.56

y = cumulative % surviving

Fig 11. Survival in patients treated with hormones alone (i.e. patients in whom the tumour responded to primary hormone therapy) in low MIB-1 (<0.4) and high MIB-1 (≥0.4) disease.

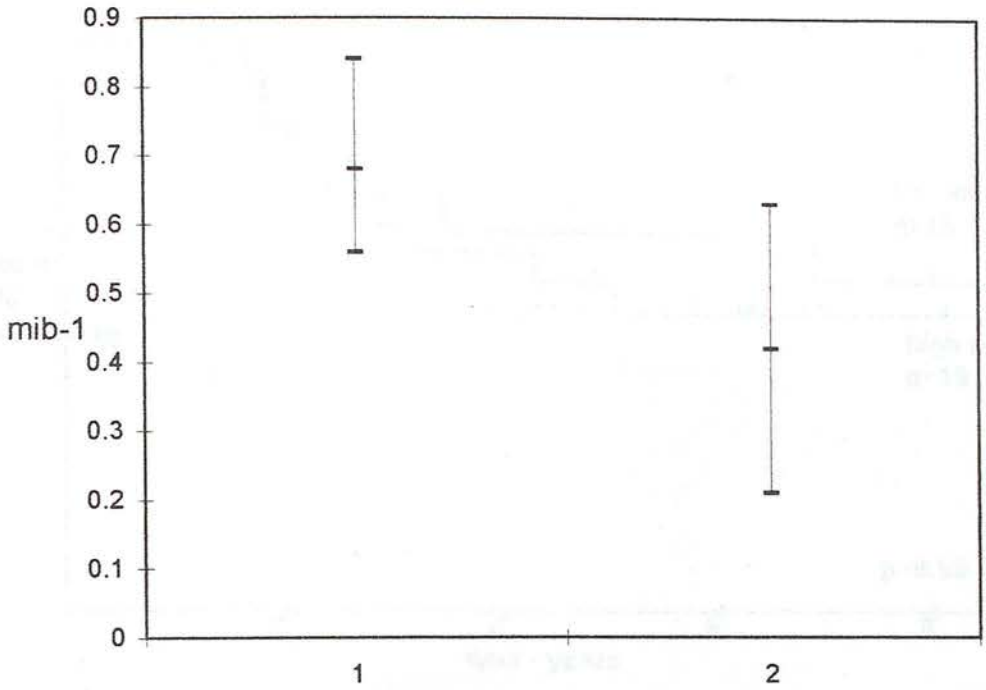


y= cumulative % surviving

p = 0.05 log rank.

median survival in low MIB-1 disease - not yet reached
median survival in high MIB-1 disease - 3 years

Fig 12. Patients treated with second line chemotherapy (after hormone failure) in low (<0.4) and high (≥0.4) MIB-1 disease.



complete pathological response

"regression" or no change

mean ± 95% confidence limits

0.68 ± 0.16

0.42 ± 0.21

$p < 0.01$ Mann-Whitney

Fig 13. MIB-1 values in patients treated with primary chemotherapy who achieved complete pathological response v those who did not.

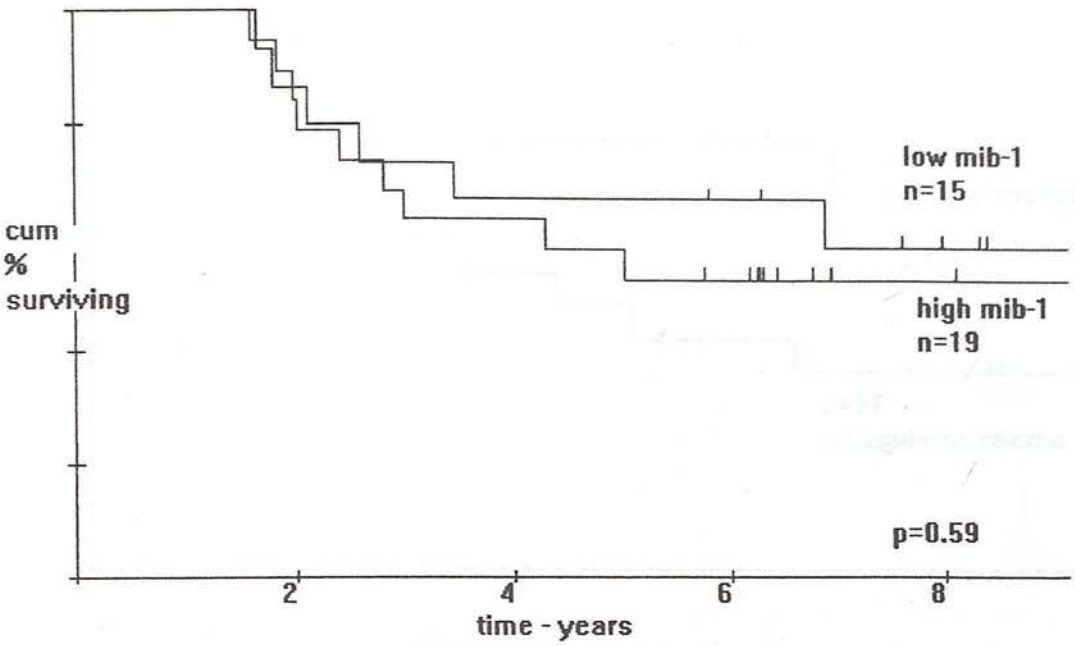
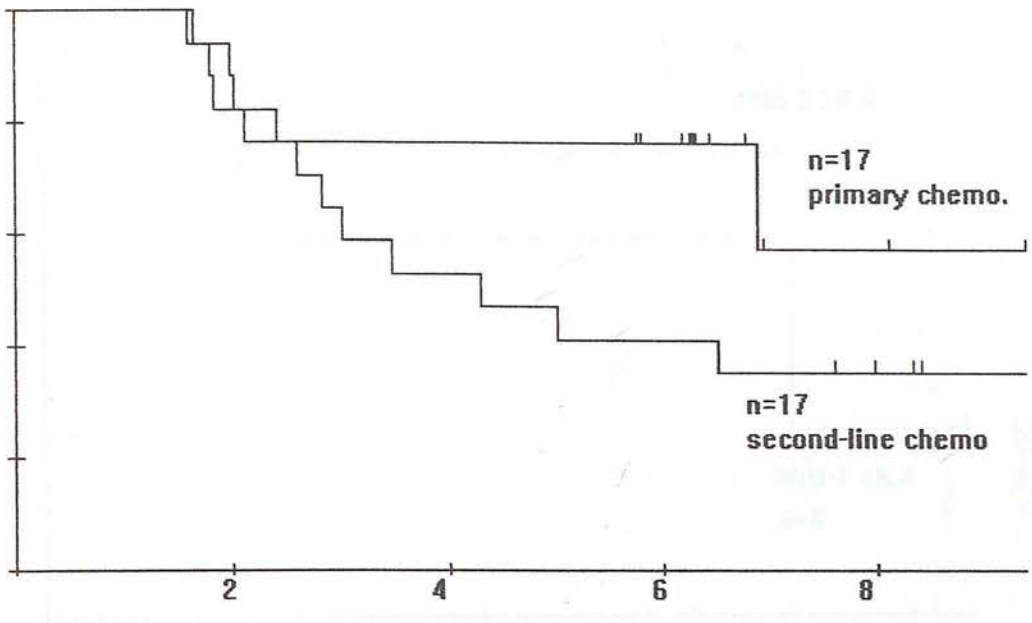


Fig 14. Survival following chemotherapy (primary or after hormone failure) in low (<0.4) and high (≥0.4) MIB-1 disease.

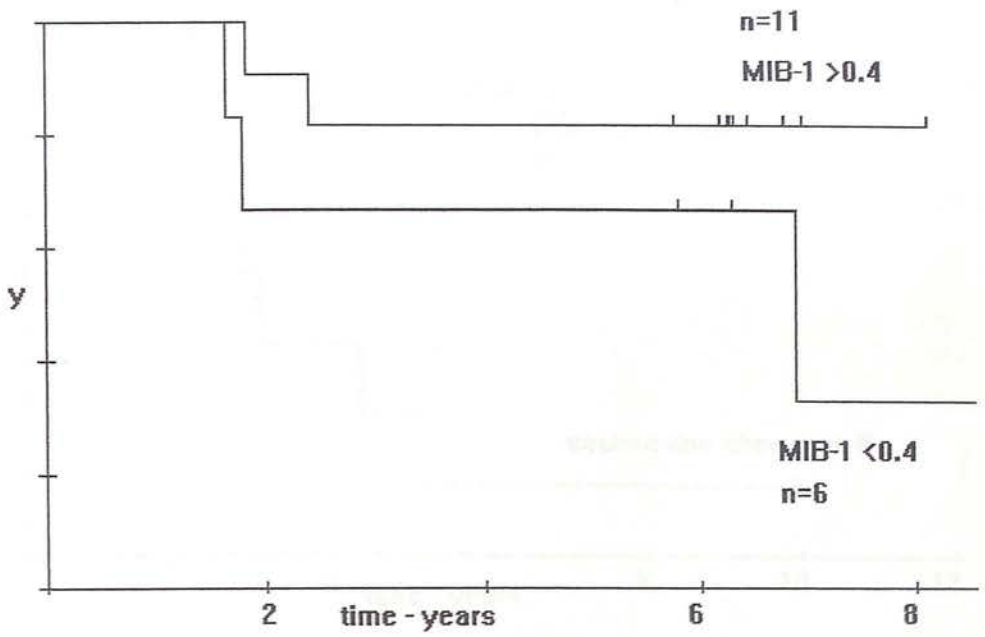


time - years

p = 0.12

median survival after primary chemotherapy not yet reached
median survival after 2nd line chemotherapy 4.3 years

Fig 15. Survival in patients treated with either primary chemotherapy or chemotherapy following primary hormone failure.

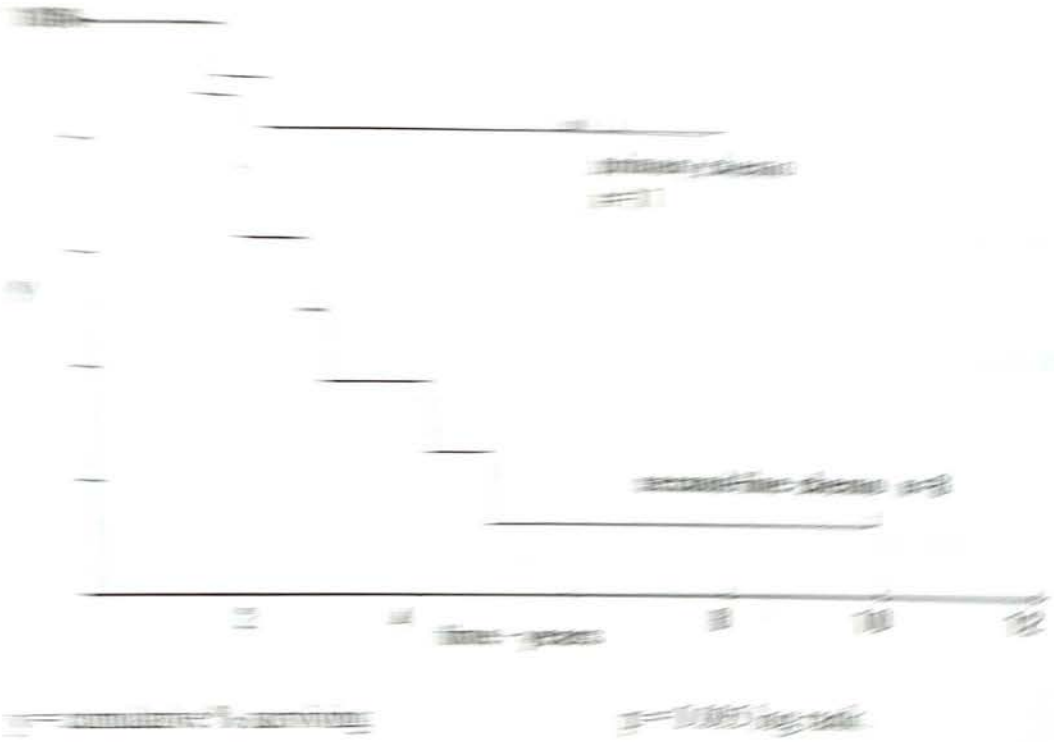


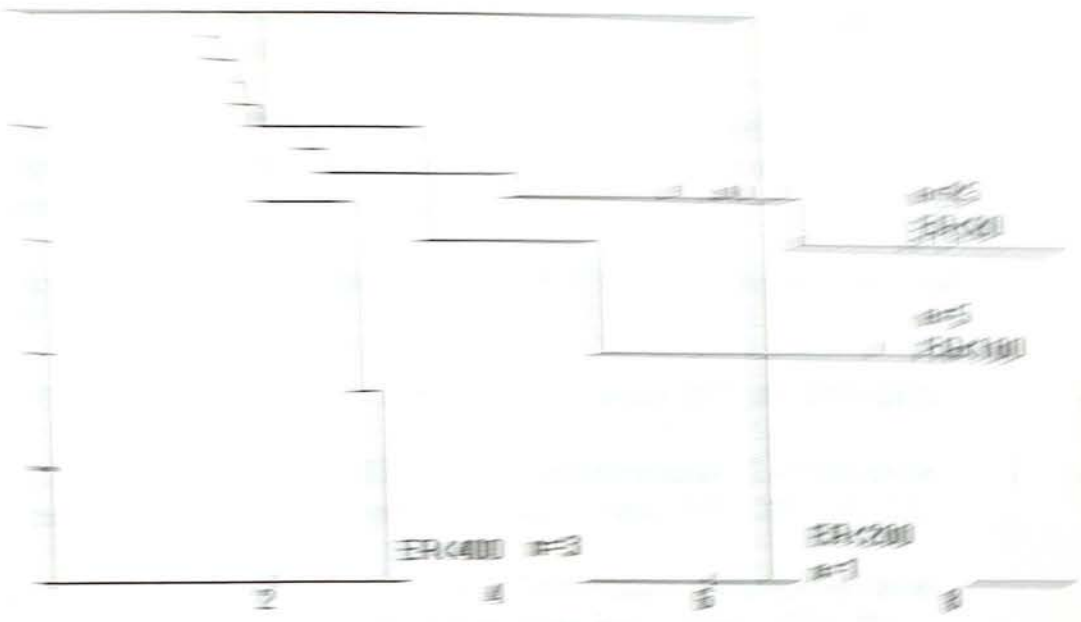
y = cumulative % surviving

p=0.23 log rank

median survival in high MIB-1 disease = not yet reached
median survival in low MIB-1 disease = 7 years

Fig 16. Survival following primary chemotherapy in low (<0.4) and high (≥0.4) MIB-1 disease.





median survival when ER<20 fmol/mg not yet reached
ER<100 5 years
ER<200 6 years
ER<400 2.8 years

Fig 18. Survival after chemotherapy (primary or secondary) in relation to ER.

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